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Subcutaneous administration of leptin normalizes fasting plasma glucose in obese type 2 diabetic UCD-T2DM rats

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Leptin has been shown to reduce hyperglycemia in rodent models of type 1 diabetes. We investigated the effects of leptin administration in University of California, Davis, type 2 diabetes mellitus (UCD-T2DM) rats, which develop adult-onset polygenic obesity and type 2 diabetes. Animals that had been diabetic for 2 mo were treated with s.c. injections of saline (control) or murine leptin (0.5 mg/kg) twice daily for 1 mo. Control rats were pair-fed to leptin-treated animals. Treatment with leptin normalized fasting plasma glucose and was accompanied by lowered HbA1c, plasma glucagon, and triglyceride concentrations and expression of hepatic gluconeogenic enzymes compared with vehicle ($P < 0.05$), independent of any effects on body weight and food intake. In addition, leptin-treated animals exhibited marked improvement of insulin sensitivity and glucose homeostasis compared with controls, whereas pancreatic insulin content was 50% higher in leptin-treated animals ($P < 0.05$). These effects coincided with activation of leptin and insulin signaling pathways and down-regulation of the PKR-like endoplasmic reticulum (ER) kinase/eukaryotic translation inhibition factor 2α (PERK-eIF2α) arm of ER stress in liver, skeletal muscle, and adipose tissue as well as increased pro-opiomelanocortin and decreased agouti-related peptide in the hypothalamus. In contrast, several markers of inflammation/immune function were elevated with leptin treatment in the same tissues ($P < 0.05$), suggesting that the leptin-mediated increase of insulin sensitivity was not attributable to decreased inflammation. Thus, leptin administration improves insulin sensitivity and normalizes fasting plasma glucose in diabetic UCD-T2DM rats, independent of energy intake, via peripheral and possibly centrally mediated actions, in part by decreasing circulating glucagon and ER stress.

glucagon reduction | lipid lowering

Leptin is an adipocyte hormone (1) that plays a pivotal role in the regulation of food intake and energy expenditure (2). Accordingly, the discovery of leptin in 1994 (1) was met with high expectations and the hope that administration of exogenous leptin might be a useful therapeutic tool for controlling obesity. However, most obese humans are not leptin-deficient (3) and are only minimally responsive to the effects of leptin to produce weight loss (4). This lack of efficacy has been attributed to resistance to the central effects of leptin to decrease food intake and body adiposity (5, 6). However, further studies of the metabolic effects of leptin in conditions in which circulating leptin concentrations are low reveal the potential for leptin to be used as a treatment for insulin resistance, hyperglycemia, and hypertriglyceridemia in models of lipodystrophy (7, 8) and type 1 diabetes (9–11).

Leptin has been shown to influence glucose homeostasis. For example, genetic leptin deficiency results in not only marked hyperphagia and obesity (1) but also severe insulin resistance, which is reversed by administration of exogenous leptin (1, 12, 13). Furthermore, leptin deficiency has been observed in models of both untreated type 1 and type 2 diabetes (9, 14, 15), and low leptin levels have been shown to contribute to the development of hyperphagia (15) and insulin resistance (9).

Several studies have demonstrated marked improvement of glucose homeostasis with systemic leptin administration in rodent models of type 1 diabetes, independent of changes of energy intake or circulating insulin concentrations (11, 16, 17). Leptin has several actions that could contribute to glucose lowering in type 1 diabetes. First, leptin administration lowers circulating glucagon levels and decreases gluconeogenic gene expression in animals with type 1 diabetes (11). Second, leptin signals to skeletal muscle to increase fatty acid oxidation, resulting in lower tissue triglyceride (TG) accumulation (18). Third, leptin administration improves insulin sensitivity in rodent models of obesity and type 1 diabetes (13, 16, 19, 20). The mechanisms by which leptin improves insulin sensitivity remain undefined but may be mediated through direct actions of leptin on peripheral tissues, such as liver and skeletal muscle, and/or indirectly via effects in the central nervous system to activate neural or endocrine signals to the periphery (19–22).

Endoplasmic reticulum (ER) stress has been shown to play an integral role in the development of insulin resistance and diabetes (23, 24). ER stress occurs when the folding capacity of the ER is exceeded and unfolded/misfolded proteins accumulate and lead to the unfolded protein response, which is triggered by transmembrane sensors that detect unfolded proteins in the ER. These proteins are PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor (ATF) 6 (25). Activation of PERK causes activation of eukaryotic translation inhibition factor (eIF) 2α, which inhibits protein synthesis as part of an adaptive response to decrease translational demands on the ER (26). The unfolded protein response also up-regulates the synthesis of chaperone proteins, such as binding Ig protein (BiP), to assist with the increased misfolded protein load (27). Induction of BiP and increased phosphorylation of PERK and eIF2α have been shown to be early molecular markers of insulin resistance in mouse models of obesity (23). Increased ER stress may also contribute to decreased leptin sensitivity in obesity and diabetes (28). Furthermore, development of ER stress within the pancreas contributes to impairment of insulin production and β-cell apoptosis in untreated diabetes (29).

The effects of leptin administration on glucose homeostasis in polygenic models of type 2 diabetes have not been investigated because most models of type 2 diabetes have defects in leptin receptor signaling, such as the *db*/*db* mouse and the Zucker diabetic fatty (ZDF) rats (30, 31). Given that leptin receptor mutations are rare in human populations and that human obesity and type 2 diabetes are most often polygenic in origin, models such as the

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db/db mouse and ZDF rat are not representative of the pathogenesis of type 2 diabetes in humans. In the present study, the effects of leptin treatment of type 2 diabetes were investigated in the University of California, Davis, type 2 diabetes mellitus (UCD-T2DM) rat model, which develops adult-onset polygenic obesity, insulin resistance, and marked hyperglycemia without a defect in leptin signaling (14). Twice-daily s.c. administration of murine leptin normalized fasting plasma glucose concentrations in diabetic UCD-T2DM rats. The normalization of fasting glucose was associated with decreased circulating glucagon concentrations, decreased expression of gluconeogenic enzymes, decreased circulating lipids, and improved insulin sensitivity. Furthermore, ER stress was decreased after leptin administration in several insulin target tissues, which likely contributed to the effects of leptin to improve insulin sensitivity and reduce hyperglycemia. These data support the utility of leptin in combination with weight loss, insulin, and/or other antihyperglycemic medications in the treatment of type 2 diabetes mellitus.

Results

Leptin Treatment Normalizes Fasted Plasma Glucose Concentrations Independent of Food Intake, Body Weight, and Circulating Insulin Concentrations. Animals that had been diabetic for 2 mo were treated with s.c. injections of saline (control) or murine leptin (0.5 mg/kg) twice daily for 1 mo. To prevent the confounding effects of leptin administration on body weight and food intake, control rats were pair-fed to leptin-treated rats (Fig. 1*A*). Average daily food intake during the month of treatment was 42.2 ± 1.7 g/d and 40.8 ± 1.7 g/d in control and leptin-treated animals, respectively. However, despite similar levels of food intake, control animals exhibited a greater loss of body weight relative to leptin-treated animals after 1 mo of treatment (control: −12 ± 2%; leptin: −5 ± 1%; $P < 0.01$). Thus, leptin-treated animals were able to more effectively maintain their body weight than the pair-fed controls were. The greater degree of weight loss in control animals was likely the result of greater energy loss from urinary glucose excretion. We have previously demonstrated that untreated diabetic UCD-T2DM rats develop progressive glucosuria (14). Plasma leptin concentrations measured at 12 h after the last leptin injection at 1 mo of treatment were 13.6 ± 4.8 ng/mL and 1.3 ± 0.6 ng/mL in leptin-treated and control animals, respectively ($P < 0.001$) (Fig. 1*C*).

After 1 mo of treatment, fasting plasma glucose concentrations were three times higher in control animals compared with leptin-treated animals (Fig. 1*D*, $P < 0.001$). Furthermore, fasting plasma glucose concentrations in leptin-treated animals were decreased by 50% compared with pretreatment values and averaged 121 ± 15 mg/dL ($P < 0.05$), close to levels observed in nondiabetic animals. Similarly, circulating HbA1c concentrations were significantly lower in leptin-treated animals compared with control animals ($P < 0.05$), and leptin-treated animals demonstrated a 2% decrease of HbA1c compared with pretreatment values ($P < 0.05$) (Table 1). Fasting plasma insulin concentrations did not differ between leptin- and vehicle-treated rats; however, fasting plasma insulin concentrations decreased in leptin-treated animals compared with baseline values, suggesting an improvement of insulin sensitivity ($P < 0.05$) (Table 1).

Leptin Treatment Decreases Circulating Glucagon Concentrations and Gluconeogenic Enzymes and Improves Lipid Metabolism. Fasting plasma glucagon concentrations were measured to determine whether leptin-mediated suppression of glucagon was present and could contribute, at least in part, to the improvement of hyperglycemia. After 1 mo of treatment, fasting plasma glucagon concentrations were lower in leptin-treated compared with control animals ($P < 0.05$) (Fig. 2*A*). Also, fasting plasma glucagon concentrations were elevated from baseline values in control animals ($P < 0.01$) but not in animals that received leptin. Furthermore,

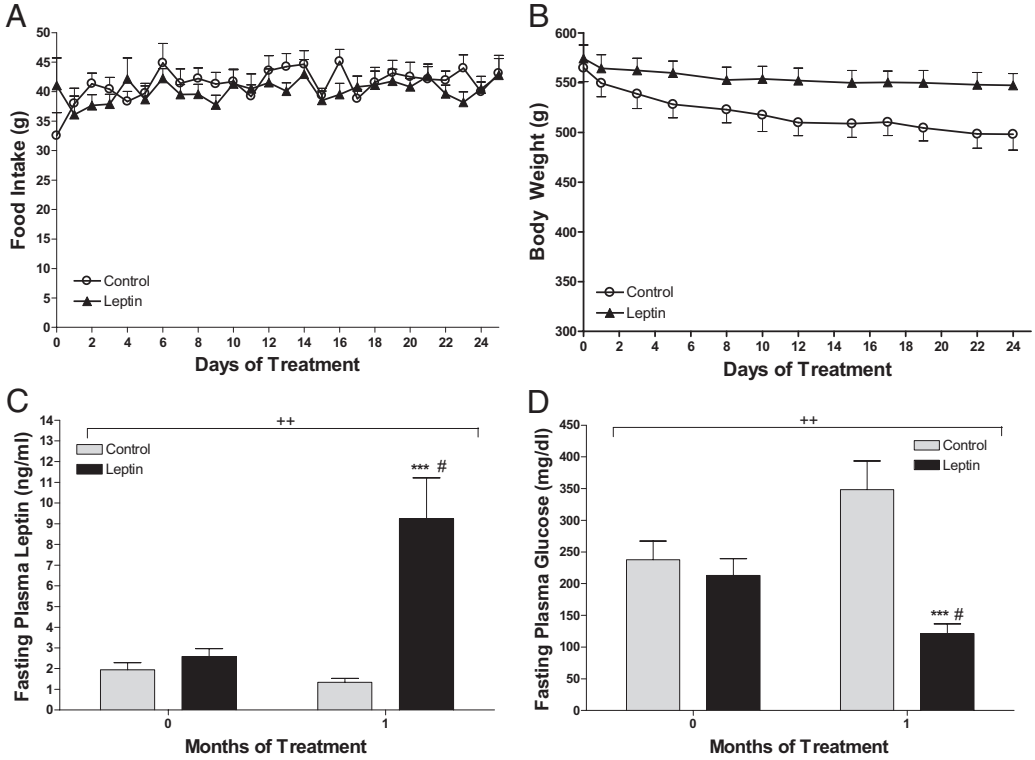

Fig. 1. Chronic leptin treatment normalizes fasting plasma glucose independent of food intake and body weight. Shown are food intake (*A*), body weight (*B*), fasting plasma leptin (*C*), and fasting plasma glucose (*D*) in diabetic UCD-T2DM rats injected with saline or leptin (0.5 mg/kg SC) twice daily for 1 mo. $^{++}P <$ 0.01 by two-way ANOVA; $^{***}P < 0.001$ compared with control by Bonferroni's posttest; and $^{\#}P < 0.05$ compared with baseline by Student's *t* test. Values are expressed as mean ± SEM.

Table 1. Plasma hormones and metabolites, tissue TG content, and pancreatic insulin and glucagon content

	Control		Leptin	
Analyte	Baseline	1 mo	Baseline	1 mo
HbA1c, %	11.0 ± 0.6	11.0 ± 1.0	10.3 ± 0.7	8.5 ± 0.4*,#
Fasting plasma insulin, ng/mL	0.7 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.6 ± 0.1#
Fasting plasma IGF1, ng/mL	855 ± 108	649 ± 74#	891 ± 84	791 ± 70
Fasting plasma TG, mg/dL	115 ± 4	144 ± 9#	123 ± 5	92 ± 7***,##
Fasting plasma cholesterol, mg/dL	104 ± 8	115 ± 7	112 ± 8	107 ± 6
Fasting plasma corticosterone, ng/mL	125 ± 21	191 ± 32#	118 ± 20	188 ± 24#
Fasting plasma adiponectin, μg/mL	1.9 ± 0.2	2.2 ± 0.2	2.0 ± 0.1	1.9 ± 0.1
Liver TG, mg/g liver	ND	4.9 ± 0.5	ND	6.1 ± 0.9
Skeletal muscle TG, mg/g of muscle	ND	2.6 ± 0.4	ND	2.6 ± 0.5
Pancreatic insulin, μg/g of pancreas	ND	7.4 ± 1.5	ND	11.2 ± 1.5*
Pancreatic glucagon, ng/g of pancreas	ND	934 ± 82	ND	1122 ± 113

Values are mean ± SEM ($n = 11$). *$P < 0.05$ and ***$P < 0.001$ compared with control by two-way ANOVA; #$P < 0.05$ and ##$P < 0.01$ compared with baseline by Student's t test. ND, not determined.

fasting plasma glucagon concentrations at the beginning of the insulin tolerance testing (ITT) were ∼50% lower in the leptin-treated compared with control animals (control: 125 ± 27; leptin: 60 ± 5 pg/mL; $P < 0.05$) and did not significantly change from baseline during the ITT in either group. Correspondingly, phospho*enol*pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) hepatic protein and mRNA were significantly lower in leptin-treated compared with control animals (Fig. 2 *B* and *C*), reflecting a potential decrease in hepatic gluconeogenesis in leptin-treated animals. Of note, pancreatic glucagon content did not differ between treatment groups (Table 1).

Fasting plasma TG concentrations in leptin-treated animals were lower compared with control animals and were decreased by 25% from pretreatment values ($P < 0.001$) (Table 1). However, fasting plasma cholesterol, liver and skeletal muscle TG content, and adipose depot weights did not differ between groups (Table 1 and Table S1).

Leptin Treatment Improves Glucose Homeostasis and Insulin Sensitivity. To directly assess insulin sensitivity, animals were subjected to ITT. Leptin-treated animals exhibited significantly greater reduction of blood glucose after insulin injection compared with controls (Fig. 2*D*). Plasma glucose concentrations in vehicle-treated animals decreased from a baseline value of 546 ± 47 mg/dL to 451 ± 108 mg/dL by 90 min after insulin administration. In contrast, plasma glucose concentrations in leptin-treated animals decreased from 432 ± 22 mg/dL to 80 ± 4 mg/dL (Fig. 2*E*). In addition to improved insulin sensitivity, leptin-treated animals demonstrated improved glucose homeostasis during oral glucose tolerance testing (OGTT), likely related to the decrease of fasting plasma glucose (Fig. 2*F*). There were no significant differences in insulin concentrations between leptin-treated and control animals during the OGTT (Fig. 2*G*). Thus, leptin administration leads to improved insulin sensitivity and enhanced glucose homeostasis in UCD-T2DM rats.

Plasma Insulin-Like Growth Factor 1 (IGF1) and Corticosterone. IGF has been shown to be elevated after leptin treatment and has been proposed to contribute to improved insulin signaling (11). Plasma IGF1 concentrations were decreased by ∼35% in control animals compared with pretreatment values ($P < 0.05$), but plasma IGF1 concentrations did not differ between groups (Table 1). Thus, IGF1 does not appear likely to have a major role in the improvement of insulin sensitivity in response to leptin treatment in UCD-T2DM rats.

Corticosterone has been suggested to contribute to the glucose-lowering effects of central leptin signaling because glucocorticoid action increases hepatic glucose production (32) and decreases insulin sensitivity (33). However, corticosterone concentrations did not differ between groups ($P < 0.05$) (Table 1). Thus, changes of corticosterone do not appear to contribute to the glucose-lowering effects of leptin in UCD-T2DM rats.

Activation of Leptin and Insulin Signaling Pathways. To investigate the molecular basis for enhanced insulin sensitivity in leptin-treated animals, we analyzed leptin and insulin signaling pathways in peripheral tissues and leptin signaling in the central nervous system of fasted control and leptin-treated animals. Janus kinase 2 (Jak2) and signal transducer and activator of transcription 3 (STAT3) tyrosyl phosphorylation was two- to threefold greater in liver, skeletal muscle, and mesenteric adipose tissue in leptin-treated animals compared with control animals, indicating activation of leptin signaling in these tissues, which may in turn contribute to the observed improvements of insulin sensitivity (Fig. 3). STAT3 phosphorylation was threefold higher in the pancreas of leptin-treated animals compared with control animals ($P < 0.01$); however, Jak2 phosphorylation in the pancreas tissue did not differ between groups (Fig. 3).

Hypothalamic agouti-related peptide (AgRP) mRNA was 40% lower ($P < 0.05$) and pro-opiomelanocortin (POMC) mRNA was sevenfold higher in leptin-treated compared with control animals ($P = 0.055$, Fig. 3*C*). These results strongly suggest that the peripherally administered leptin reached the central nervous system and activated leptin-mediated signal-transduction pathways.

Activation of Akt and MAPK was significantly elevated in several insulin-sensitive peripheral tissues. Phosphorylation of Akt was increased by two- to threefold in mesenteric adipose, liver, skeletal muscle, and pancreas (Fig. 3). In addition, phosphorylation of MAPK was increased by 78% in mesenteric adipose, 30% in skeletal muscle, and 62% in pancreas compared with control animals (Fig. 3).

Leptin Treatment Decreases the PERK-eIF2α Arm of ER Stress. ER stress has been shown to play an integral role in the development of insulin resistance. Thus, we measured markers of ER stress to determine whether the leptin-mediated improvement of insulin sensitivity corresponded with an improvement of ER stress. Markers of the PERK pathway of ER stress were significantly lower in liver, skeletal muscle, mesenteric adipose, and pancreas tissue after 1 mo of leptin treatment compared with vehicle-treated animals. Phosphorylation of PERK and eIF2α was reduced by ∼83% in liver, ∼73% in skeletal muscle, ∼63% in mesenteric adipose, and ∼40% in pancreas tissue of leptin-treated animals compared with controls (Fig. 4). Furthermore, BiP expression was reduced by 36% in liver, 71% in skeletal muscle, 40% in mesenteric adipose, and 64% in pancreas tissue of leptin-treated animals compared with controls (Fig. 4). Pan-

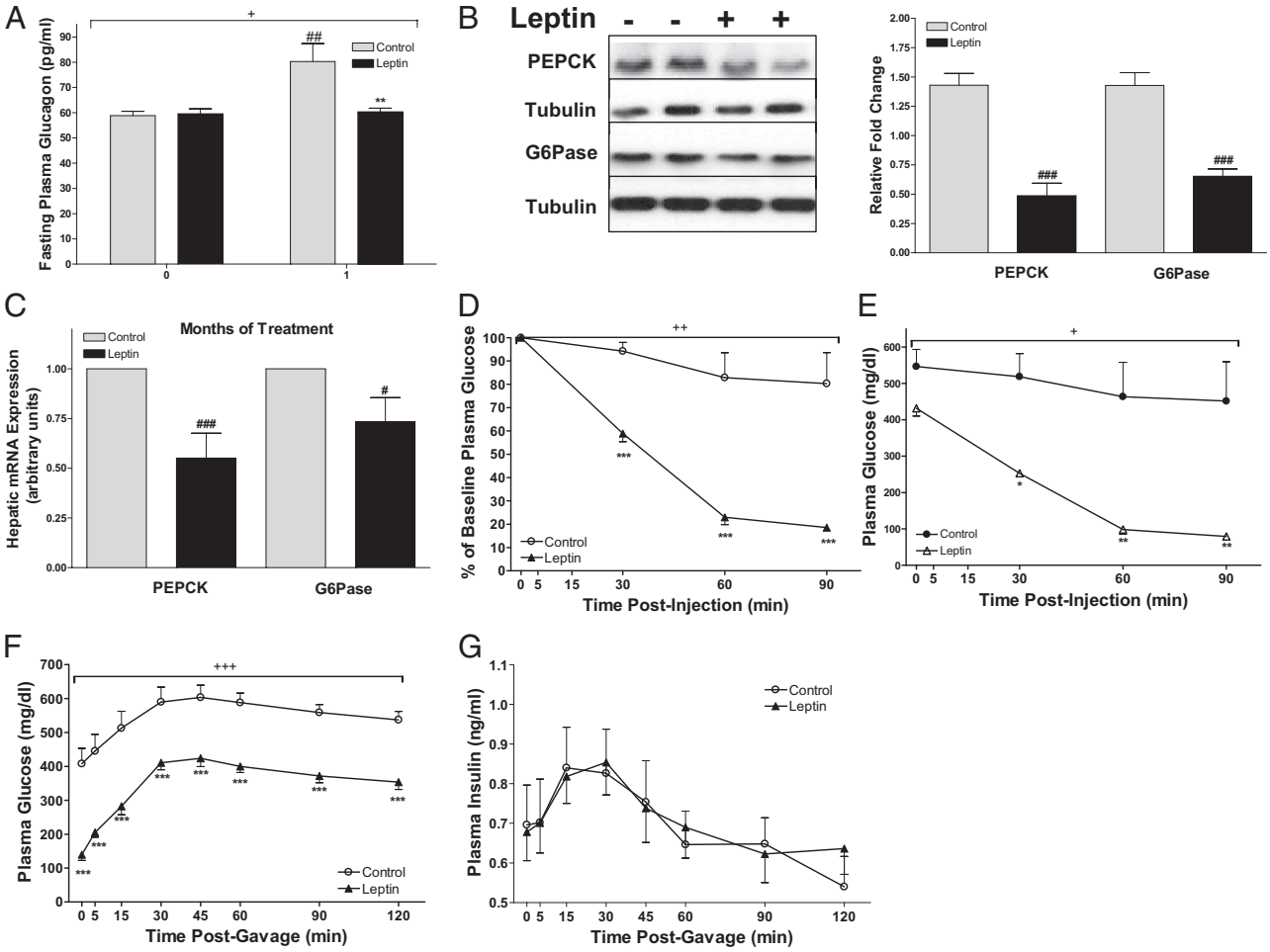

Fig. 2. Chronic leptin treatment improves glucose homeostasis and insulin sensitivity. (*A*) Fasting plasma glucagon (13-h fast) at 1 mo after initiation of leptin treatment ($n = 11$ per group). (*B* and *C*) Hepatic PEPCK and G6Pase protein (*B*) and mRNA expression (*C*) at 1 mo after initiation of leptin treatment ($n = 11$ per group). (*D* and *E*) Percentage of baseline plasma glucose (*D*) and plasma glucose concentrations (*E*) during an ITT preformed 2 wk after the initiation of leptin treatment ($n = 3$ per group). Animals were fasted for 4 h and then injected with 1 U/kg regular insulin. (*F* and *G*) Glucose (*F*) and insulin (*G*) during an OGTT at 1 mo after initiation of leptin treatment ($n = 11$ per group). Animals were fasted overnight and gavaged with 1 g/kg of glucose. $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ by two-way ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control by Bonferroni's posttest; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ by Student's *t* test. Values are expressed as mean ± SEM.

creatic insulin content was significantly greater in leptin-treated animals compared with controls after 1 mo of treatment ($P < 0.05$) (Table 1), which could be related to decreased ER stress in the pancreas.

We hypothesized that the decrease of ER stress, especially in adipocytes, may cause an increase in adiponectin production, which could contribute to the improvement of insulin sensitivity. However, circulating adiponectin concentrations did not differ between groups (Table 1).

In contrast to the improvement of insulin sensitivity and ER stress response, 1 mo of leptin treatment resulted in increased protein levels of markers of inflammation/immune function, including monocyte chemotactic protein 1 (MCP-1) and TNF-α in liver, skeletal muscle, and mesenteric adipose tissue (Fig. S1).

Discussion

In the present study, we investigated the efficacy of leptin in the treatment of type 2 diabetes in UCD-T2DM rats, an animal model of polygenic adult-onset obesity, insulin resistance, and inadequate β-cell compensation leading to type 2 diabetes (12). Twice-daily leptin administration (0.5 mg/kg) normalized fasting plasma glucose concentrations independent of differences in food intake or plasma insulin concentrations. Fasting plasma leptin concentrations achieved with twice-daily administration of 0.5 mg/kg of leptin were similar to those observed in prediabetic UCD-T2DM rats (14). Among the effects of leptin that are likely to have contributed to the normalization of fasting plasma glucose are decreased hepatic glucose production and marked improvement of insulin sensitivity. In addition, leptin-mediated improvement of insulin sensitivity and hyperglycemia coincided with decreased ER stress. This study demonstrates a beneficial effect of leptin treatment to reduce hyperglycemia in an obese, polygenic model of type 2 diabetes.

Similar to previous studies in models of type 1 diabetes, fasting plasma glucagon concentrations were significantly reduced in leptin-treated animals (9, 11, 17, 34). This reduction of glucagon led to decreased hepatic PEPCK and G6Pase mRNA and protein, which may have resulted in a decrease of hepatic gluconeogenesis in leptin-treated animals and therefore to the improvement of fasting plasma glucose concentrations in leptin-treated animals. The increased pancreatic insulin content in leptin-treated animals suggests that glucagon secretion was decreased in leptin-treated animals as a paracrine effect of insulin to restrain glucagon release from pancreatic α-cells (35).

Leptin treatment potently improved insulin sensitivity, as demonstrated by the increased response to insulin during an ITT and increased activation of insulin signaling in liver, skeletal muscle, and mesenteric adipose tissue. These observations are in

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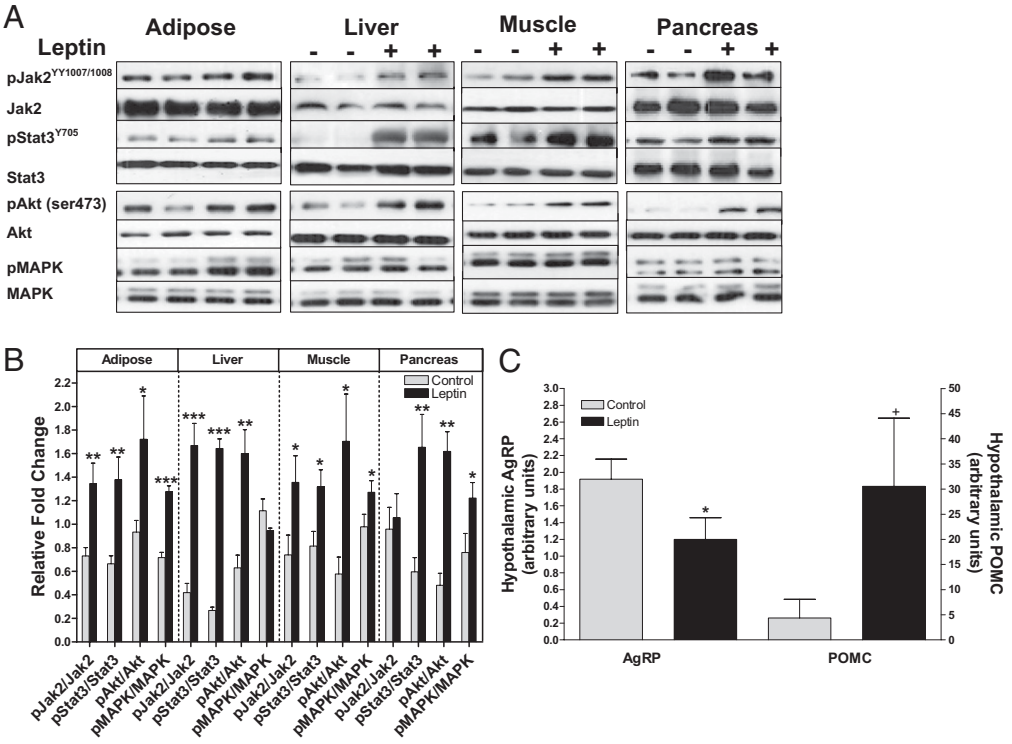

Fig. 3. Chronic leptin treatment up-regulates the leptin and insulin signaling pathways. Rats received their final injection and were fasted overnight, and tissues were collected the following day. (*A*) Immunoblotting for phosphorylated (pJak2) and total Jak2, phosphorylated (pSTAT3) and total STAT3 (*Upper*), phosphorylated (pAkt) and total Akt, and phosphorylated (pMAPK) and total MAPK (*Lower*) in adipose, liver, muscle, and pancreas tissue. (*B*) All blots were scanned and quantified with FluorChem 9900. Results were quantified in densitometric units and expressed relative to the total protein of interest. (*C*) Hypothalamic mRNA expression of AgRP and POMC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{+}P = 0.055$ compared with control by Student's *t* test. ($n = 6$ per group for immunoblots; $n = 4$ per group for hypothalamic mRNA.) Values are expressed as mean ± SEM. Bar graphs represent normalized data.

agreement with several previous studies (9, 16, 17, 19). Improvement of insulin sensitivity with leptin treatment has been demonstrated to be mediated both directly by peripheral leptin signaling and indirectly by leptin signaling to the hypothalamus and hindbrain (11, 19–22). We observed marked increases of Jak2 and STAT3 phosphorylation in liver, skeletal muscle, and adipose tissue of leptin-treated animals compared with control animals. These data suggest that leptin is signaling directly to peripheral tissues to improve insulin sensitivity. Direct signaling to peripheral tissues and subsequent phosphorylation of Jak2 could contribute to improved insulin signaling because Jak2 can activate PI3K, a downstream mediator of insulin signaling (36). However, these results do not exclude the possibility that central leptin signaling also contributed to the improvement of insulin sensitivity (10, 11, 17, 19, 34, 37). Furthermore, the decrease of AgRP and increase of POMC hypothalamic mRNA strongly indicates a component of central leptin signaling in this study that likely contributed to the improvement of peripheral insulin sensitivity.

ER stress has been shown to play an important role in the development of insulin resistance and type 2 diabetes (23, 28). Furthermore, attenuation of ER stress with administration of chemical chaperone proteins has been shown to reverse type 2 diabetes in mice (24). In this study, we show that chronic leptin administration decreased the PERK-eIF2α subarm of ER stress signaling in liver, skeletal muscle, and mesenteric adipose tissue, an effect that likely contributed to the improvement of insulin sensitivity.

In contrast, several inflammatory mediators, such as MCP-1 and TNF-α, were elevated in the liver, muscle, and adipose tissue of leptin-treated animals compared with control animals. Furthermore, the decrease of ER stress would be expected to result in a decrease of inflammation (38). However, this increase of circulating immune/inflammatory mediators may represent a normalization of immune function, rather than the induction of an inflammatory state, because leptin deficiency is associated with impaired immune function, which is restored after leptin administration (39). These data suggest that leptin-mediated decreases of ER stress improve insulin sensitivity by mechanisms other than decreasing inflammation.

Similar to previous studies, leptin treatment resulted in a marked reduction of circulating TG (17). However, liver and skeletal muscle TG content did not differ between groups. Although previous studies have shown that leptin treatment lowers tissue TG

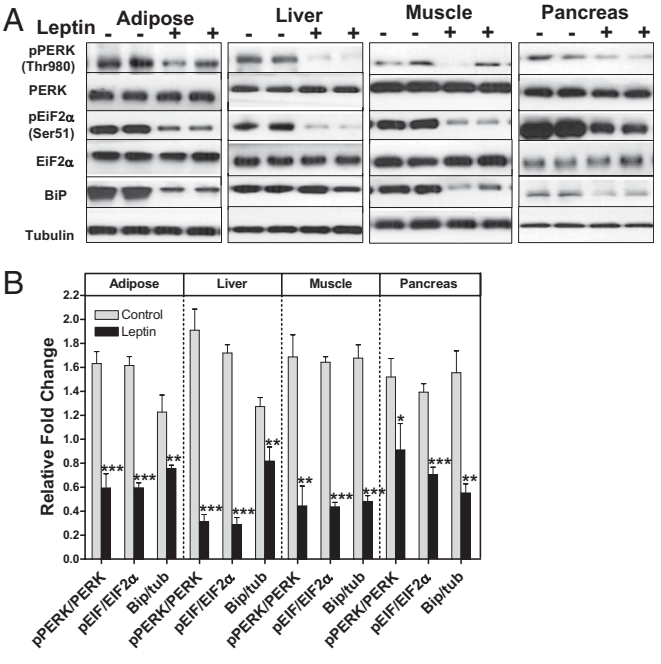

Fig. 4. Attenuated PERK-eIF2α signaling in rats with chronic leptin treatment. (*A*) Immunoblotting for phosphorylated [pPERK(Thr980)] and total PERK, phosphorylated [pEiF-2α(Ser51)] and total eIF-2α, BiP, and tubulin in adipose, liver, muscle, and pancreas tissue. (*B*) Results were quantified in densitometric units and expressed relative to the total protein of interest or relative to tubulin for BiP. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control by Student's *t* test. ($n = 6$ per group.) Values are expressed as mean ± SEM.

deposition (9), the data in this study are confounded by the pair-fed control animals having lost a greater percentage of body weight at 1 mo. The lack of differences in tissue TG deposition suggests that alterations of lipid metabolism are not likely to have a major role in the leptin-mediated improvement of insulin sensitivity in UCD-T2DM rats. However, the decreased circulating TG reflects an additional benefit of leptin administration.

Leptin treatment resulted in greater pancreatic insulin content, which may have been mediated by improvement of pancreatic insulin signaling and by a decrease of ER stress. Insulin signaling in the β-cell has been shown to induce β-cell proliferation (40), and ER stress in the β-cell has been implicated in progressive β-cell failure observed in type 2 diabetes (29). However, because these measurements were made in whole pancreas tissue, it is difficult to ascribe the decreases of ER stress specifically to the islet component of the pancreas.

In conclusion, chronic treatment with leptin normalizes fasting plasma glucose in an animal model of polygenic obesity and type 2 diabetes. Decreases of circulating glucagon concentrations and increased insulin sensitivity mediated by decreased ER stress are likely contributors to the improvement of glucose homeostasis. Overall, these results suggest that leptin administration could be useful in the treatment of type 2 diabetes by improving insulin sensitivity and common comorbidities of type 2 diabetes mellitus such as hypertriglyceridemia.

Materials and Methods

Diets and Animals. Male UCD-T2DM rats were housed in the University of California, Davis, Department of Nutrition animal facility and maintained on a 14-h light:10-h dark cycle. At the time of intervention, animals were 6 mo old and had been diabetic for 2.1 ± 0.1 mo (n = 11 per group). Diabetes onset was defined as a nonfasted blood glucose >200 mg/dL measured (between 1400 and 1600 hours) for 2 wk consecutively in ad libitum-fed colony animals. Baseline nonfasting blood glucose was 540 ± 11 mg/dL and 526 ± 11 mg/dL in control and leptin groups, respectively. Animals received ground rodent chow (no. 5012; Ralston Purina). Control animals were pair-fed to leptin-treated animals.

Control animals received s.c. saline injections (1 mL/kg), and leptin-treated animals received s.c. murine recombinant leptin injections (0.5 mg/kg) twice daily (0800 and 1800 hours). An ITT was conducted on a subset of three animals per group after 2 wk of treatment. Animals were fasted for 4 h and then received an i.p. injection of 1 U/kg regular insulin (Novolin R). After 1 mo of treatment, animals received their last injection and were fasted overnight and an OGTT was conducted the following morning. Animals received a 50% glucose solution (1 g/kg) by oral gavage, and blood was collected from their tails. Animals were then euthanized 4 h later with an overdose of pentobarbital (200 mg/kg i.p.). Tissues were dissected, weighed, flash-frozen in liquid nitrogen, and stored at −80 °C. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Standard methods for plasma and tissue analyses are described in detail in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Hormone and Metabolite Profiles. Blood samples were collected from animals the day before the start of treatment and at 1 mo after treatment initiation. Animals were fasted overnight (13 h), and samples were collected into EDTA-treated tubes. Plasma glucose, cholesterol, and TG were measured with enzymatic colorimetric assays (Thermo DMA). Leptin, glucagon, and adiponectin were measured with rodent-specific RIAs (Millipore). Insulin and IGF1 were measured by rat-specific ELISAs (Millipore and R&D Systems). HbA1c was measured with an enzymatic colorimetric assay (Diazyme), and corticosterone was measured by RIA (MP Biomedical).

Pancreatic Hormone Content and Tissue TG Content. Pancreas insulin and glucagon extraction was performed as previously described (1). Liver and skeletal muscle TG content were measured by using the Folch method for lipid extraction (2) followed by spectrophotometric measurement of TG content (Thermo Electron).

Immunoblotting. Tissues were ground in liquid nitrogen and lysed with radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 7.4) plus 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors]. Lysates were clarified by centrifugation at 15,000 × *g* for 10 min, and protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Chemical). Proteins were resolved by SDS/PAGE and transferred to nitrocellulose membranes. Immunoblots were performed with indicated antibodies (Table S2). Proteins were visualized with enhanced chemiluminescence (ECL; Amersham Biosciences), and pixel intensities of immunoreactive bands were quantified with FluorChem 9900 (Alpha Innotech).

Quantitative Real-Time PCR. Hepatic mRNA was extracted with TRIzol reagent (Invitrogen), and hypothalamic mRNA was extracted with TRIzol B (MRC\). cDNA was generated by using the Reverse Transcription System (Promega). mRNA expression was assessed by quantitative real-time PCR (hepatic: iCycler; Bio-Rad; hypothalamic: ABI Prism 7900 HT; Applied Biosystems) with the appropriate primers (Table S3). Hepatic mRNA was normalized to GAPDH, and hypothalamic mRNA was normalized to 18S RNA.

Statistics and Data Analyses. Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 4.00 for Windows (GraphPad Software). Body weight, food intake, and fasting hormone and metabolite data and ITT and OGTT data were compared by two-factor (time and treatment) repeated-measures ANOVA followed by post hoc analysis with Bonferroni's multiple-comparison test. Tissue weights, tissue TG content, pancreatic hormone content, tissue protein, mRNA expression, and changes from baseline in plasma hormones and metabolites were analyzed with Student's *t* test. Differences were considered significant at $P < 0.05$.

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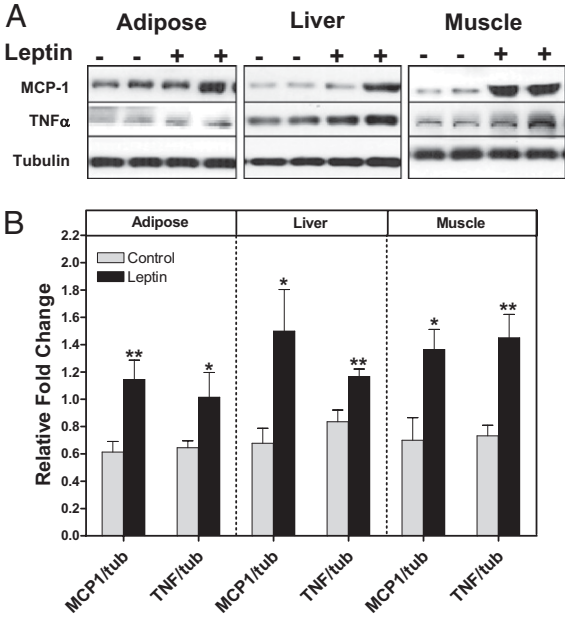

Fig. S1. Chronic leptin treatment increases markers of inflammation in peripheral tissues. (*A*) Immunoblotting for MCP-1, TNF-α, and tubulin in adipose, liver, and muscle tissue. (*B*) Results were quantified in densitometric units, and MCP-1 and TNF-α were expressed relative to tubulin. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with control by Student's *t* test. ($n = 6$ per group.) Values are expressed as mean ± SEM.

Table S1. Tissue weights after 1 mo of leptin treatment

Tissue	Control	Leptin
Epididymal adipose depot	3.4 ± 0.5	3.9 ± 0.5
Retroperitoneal adipose depot	4.0 ± 0.7	4.6 ± 0.8
s.c. adipose depot	13.2 ± 2.5	16.4 ± 2.7
Mesenteric adipose depot	2.4 ± 0.2	2.4 ± 0.4
Total white adipose tissue	23 ± 3	27 ± 4
Heart	1.3 ± 0.1	1.4 ± 0.1
Kidney	2.2 ± 0.1	2.2 ± 0.1
Liver	17.6 ± 0.4	18.3 ± 0.4

Values are mean ± SEM (n = 11). All weights are in grams.

Table S2. Western blot antibodies

Antibody	Source	Host	Dilution for Western blot
eIF2α	Santa Cruz Biotechnology	Mouse	1:1,000
Akt	Santa Cruz Biotechnology	Mouse	1:5,000
JNK	Santa Cruz Biotechnology	Mouse	1:1,000
MAPK	Cell Signaling	Rabbit	1:5,000
MCP-1	Santa Cruz Biotechnology	Goat	1:1,000
PERK	Santa Cruz Biotechnology	Rabbit	1:1,000
Phospho-PERK (Thr980)	Santa Cruz Biotechnology	Rabbit	1:500
Phospho-Akt (Ser473)	Cell Signaling	Rabbit	1:10,000
Phospho-eIF2a (Ser51)	Santa Cruz Biotechnology	Rabbit	1:1,000
Phospho-JNK (Thr-183/Tyr-185)	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-MAPK (Thr-202/Tyr-204)	Cell Signaling	Rabbit	1:10,000
TNF-α	Santa Cruz Biotechnology	Goat	1:1,000
Tubulin	Santa Cruz Biotechnology	Mouse	1:10,000
PEPCK	Santa Cruz Biotechnology	Mouse	1:1,000
G6Pase	Santa Cruz Biotechnology	Rabbit	1:250

eIF2α, eukaryotic translation inhibition factor 2α; G6Pase, glucose-6-phosphatase; MCP-1, monocyte chemotactic protein 1; PEPCK, phospho*enol*pyruvate carboxykinase; PERK, PKR-like endoplasmic reticulum kinase.

Table S3. Real-time PCR primers

Gene	Direction	Sequence (5′ to 3′)
PEPCK	Forward	GAGGCCTCCCAACATTCAT
	Reverse	CTCAGAGCGTCTCGCCGGA
G6Pase	Forward	CAGGAGCCACACAGTTGAAACAGA
	Reverse	AGGGTGATTTACGTAAAATAGCAAA
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA
AgRP	Forward	AGGGCATCAGAAGGCCTGACCAGG
	Reverse	CATTGAAGAAGCGGCAGTAGCACGT
POMC	Forward	CGCCCGTGTTTCCA
	Reverse	TGACCCATGACGTACTTCC

AgRP, agouti-related peptide; POMC, pro-opiomelanocortin.